Supporting Information

Student Handout

Background on the ELISA module

Enzyme-linked immunosorbent assays (ELISA) are critically important tools that allow the quantitative determination of a variety of molecules including disease biomarkers, hormones, antibodies, viruses, bacteria, pollutants, etc., in biological and environmental samples.[1] The ELISA method utilizes the specific and selective interaction between an antigen and an antibody to detect one or the other in complex sample matrices (e.g., serum) without the need for much sample preparation. It is this aspect of the ELISA technique that makes it highly reliable and has led to its widespread use in many clinical, pharmaceutical, and basic scientific investigations. In a sandwich ELISA procedure as reported in this article, a solution of a capture species is first incubated in an assay chamber to passively or covalently immobilize it on the walls of this compartment. After washing out the excess material, the unreacted sites on the solid surface are then blocked with a relatively inert molecule (e.g., protein/amino acid) so as to prevent any non-specific immobilization of the undesirable sample constituents. At this point, the sample is introduced into the ELISA compartment to allow specific binding of the analyte molecules to the immobilized capture species. A detection antibody conjugated to an enzyme label is later reacted with the assay surface which again specifically binds to the captured analyte on the chamber walls making a sandwich-like biomolecular complex. The free enzyme conjugates are washed away from the assay compartment with a washing buffer following this step. The signal in the ELISA method is generated through addition of an enzyme substrate to the assay chamber which gets converted into a detectable product. The rate of generation of this product then offers a

measure for the amount of analyte present in the sample. Notice that there is often a non-zero rate of signal generation in the ELISA technique even if the sample did not contain any analyte molecules (blank assay). This occurs due to non-specific binding of the detection antibody to the chamber walls and tends to be significantly larger compared to the noise in the system. In this situation, it is necessary to subtract the blank signal from that yielded by an actual sample to accurately estimate the analyte concentration in it.

Recently, miniaturization of the ELISA method on the microfluidic platform has been shown to yield several advantages, including a reduced sample volume requirement, shorter incubation period and greater sensitivity.[2] Moreover, microfluidic ELISA platforms are inexpensive to fabricate and allow integration of analytical procedures, such as sample pre-concentration, that further enhance the performance of the immunoassay. A microfluidic device typically comprises a network of micrometer scale channels connecting different circular wells created on a substrate material such as glass or plastic. While chemical and biological assays are performed on these devices by moving fluid samples through the closed conduits, the circular wells act as control ports for manipulating this transport process. Many of the advantages listed for microfluidic ELISA arise due to the smaller dimensions of the microfabricated channels which serve as the assay chamber in these systems. In Figure 1, we have compared the typical dimensions of a microchannel to those of cylindrical microwells on a microtiter plate that are commonly used for carrying out immunoassays. As may be seen, while the volume of these conduits is about 200 nL, the corresponding quantity for the microwells is over 1000-fold larger (> 200 μL). This leads to a significant reduction in the sample volume requirement in microfluidic ELISAs as mentioned above. The surface area of the microchannels (~ 0.16 cm^2) on the other hand, turns out to be only 10-fold smaller than that of their microwell counterpart

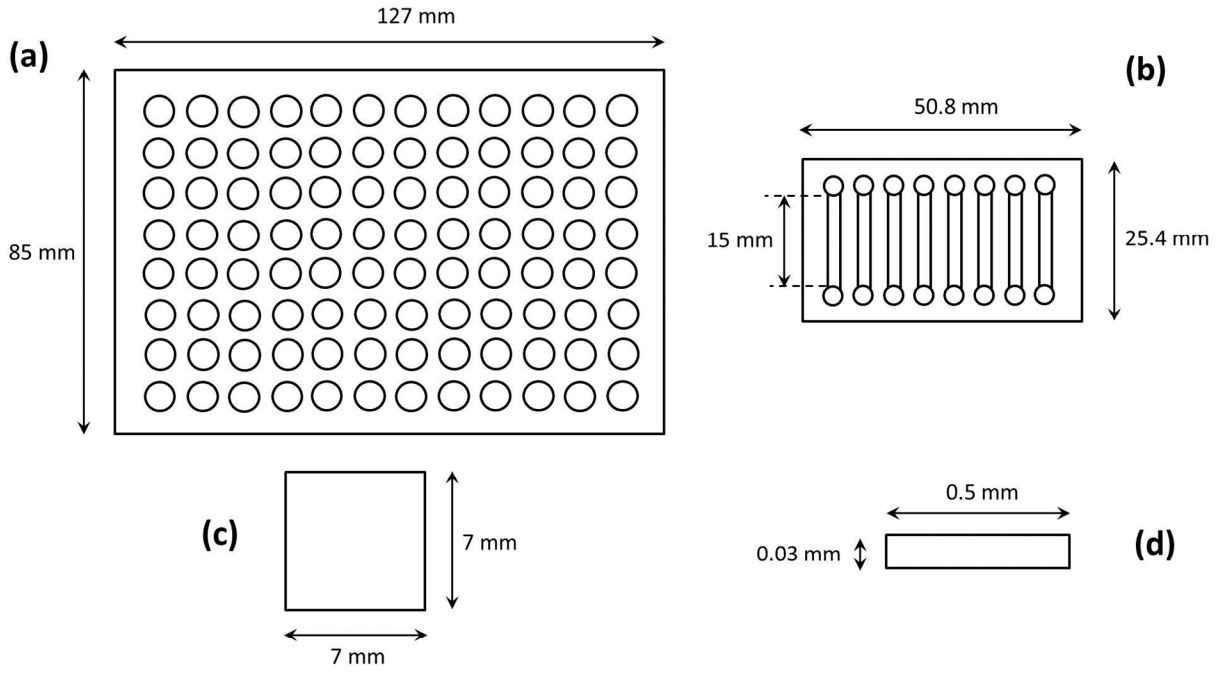

Figure 1: Comparison of the physical dimensions of a microwell to those of a microfluidic channel. Top view of a **(a)** 96-well microtiter plate **(b)** microfluidic device. Cross-sectional view of a **(c)** microwell **(d)** microfluidic channel.

resulting in substantially larger surface area to volume ratio in these systems. Moreover, the length scale over which analytes/antibodies need to diffuse to reach the assay surface in the microfabricated ducts is over 2 orders of magnitude shorter than that in microwells (0.03 mm versus 7 mm). These two factors in combination reduces the incubation period in microfluidic systems by expediting the immunocapture process, and at the same time allows the detection of smaller quantities of the analyte in the sample.

Recognizing the scientific potential of microfluidic ELISAs, the current experiment describes a laboratory procedure for introducing this bioanalytical method to an upper-division undergraduate curriculum. This major objective is accomplished by applying the immunoassay to the quantitative determination of anti-mouse BSA (bovine serum albumin) in a liquid sample

using glass microchannels. In addition, the reported module provides training on quantitating ELISAs using the kinetic format of the assay, basic image analysis techniques, as well as signal-to-noise ratio and limit of detection calculations that are valuable in characterizing any analytical method. For the current experiment, microfabricated channels etched on a glass plate using standard photolithographic and wet etching methods[3] are employed. The walls of these microchannels are sequentially reacted with (3-aminopropyl)triethoxysilane and glutaraldehyde to allow the covalent attachment of the capture species (BSA) to them (see Figure 2). BSA molecules thus immobilized on the microchannel walls are incubated with a sample containing anti-mouse BSA (analyte). The detection antibody (goat anti-mouse IgG HRP conjugate) is subsequently reacted with the assay surface to complete the sandwich-like biomolecular complex. An enzyme substrate solution containing Amplex Red and hydrogen peroxide is introduced into the ELISA microchannel allowing the quantitation of the assay through monitoring of the fluorescent resorufin species produced over the enzyme reaction period. Notice that the enzyme reaction in the current system is carried out at saturation kinetics so as to minimize any changes in its rate due to the decreasing Amplex Red/hydrogen peroxide concentration in the microchannel with time. Experiments reported in the literature suggest that the rate of signal generation in a microfluidic ELISA tends to be highly sensitive to the ambient temperature[4,5] which therefore is controlled in order to improve the reproducibility of the measurements. A simple approach to accomplishing this task is to maintain a constant air temperature (e.g. 37°C) around the microchip through placement of a heating fan close to it. Because the optical detection in the current set-up can be made without disturbing the air flow around the microchip in any significant way, no major temperature fluctuations are expected in the microchannels during the data collection process.[4,5] Fluorescence measurements at the center

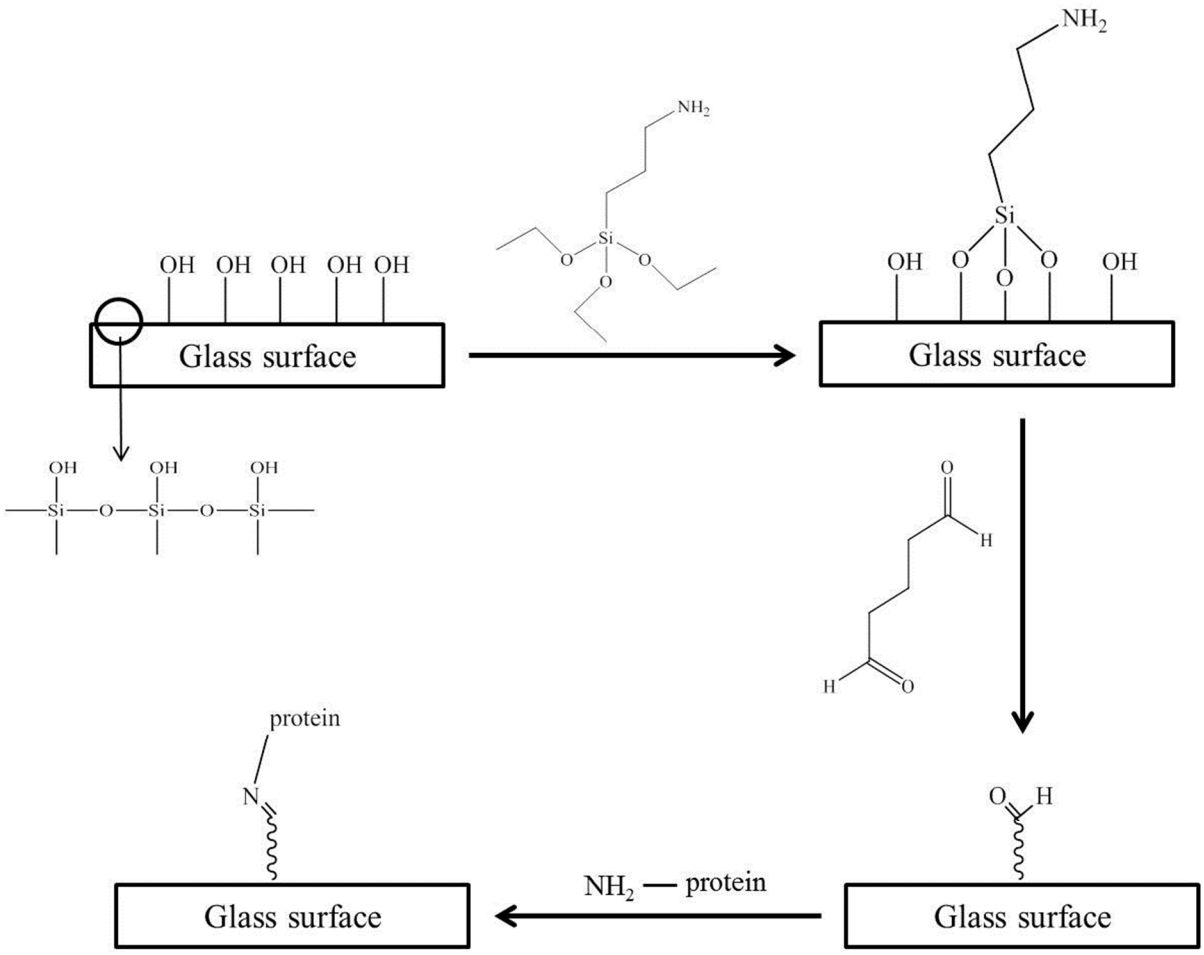

Figure 2: Schematic of the chemical reactions by which glass microchannels are to be treated in the present experimental module to allow the covalent attachment of the capture species in the ELISA method to the channel walls.

of each ELISA channel are made using an epifluorescence microscope with band-pass excitation (528-543 nm) and emission (590-650 nm) optical filters. The fluorescence images thus obtained using a CCD camera, are analyzed with Adobe Photoshop software. To minimize any unwanted signal generation in the reported system through photo-oxidation of Amplex Red,[6] the ELISA regions are exposed to the excitation beam for < 3 s during the imaging process using a mechanical shutter. It should be noted that while the actual intensity measured from the fluorescence images are dependent on the kind of camera used, its operational settings as well as

the image processing software employed, the limit of detection for the assay tends to be relatively insensitive to these variables.

References

(1) Kemeny, D. M.; Challacombe, S. J. *ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects*; John Wiley & Sons: New York, 1998.

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(4) Yanagisawa, N.; Dutta, D. Kinetic ELISA in microfluidic channels. *Biosensors* **2011**, *1*, 58-69.

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Student Procedure for Performing Microfluidic ELISA

Note: All regents to be used in this module will be prepared by the teaching assistants before the beginning of the laboratory period and will be provided to you in labeled vials.

1. Introduce a solution of 1 M sodium hydroxide (NaOH) into the microchannel ensuring that no air bubbles are trapped within it. Seal the access holes using an adhesive tape to prevent any evaporation of this liquid from the channel terminals and incubate for 10 min.

2. Remove the NaOH solution from the microchannel using a vacuum pump. In order to minimize contamination issues, it is suggested that only one of the channel terminals be used for introducing solutions and the other for draining them out. Now rinse the channel by flowing de-ionized water through it applying vacuum for 1 min. Repeat this washing step with methanol for another 1 min. Place the microchip in an oven maintained at 80°C for 5 min and later cool it to room temperature.

3. Introduce (3-aminopropyl)triethoxysilane (APTES) into the channel and seal its access holes using an adhesive tape immediately. APTES is hydrolytically unstable and can react with moisture from the atmosphere. Allow APTES to react with the surface of the glass channel for 15 min.

4. Remove the excess APTES solution and rinse the channel with methanol for 1 min. Dry the channel by placing the microchip in an oven maintained at 80°C for 5 min. and later cool it to room temperature.

5. Introduce a 5% (w/v in water) glutaraldehye solution into the conduit, tape its access holes and incubate for 15 min. Remove the excess glutaraldehyde solution and wash the channel with water for 1 min.

6. Introduce a 1% w/v BSA solution prepared in carbonate-bicarbonate buffer (0.1 M, pH 9.4), seal the access holes with adhesive tapes and incubate for 10 min. Remove the BSA solution and wash the channels with phosphate buffer (0.1 M, pH 7.4) for 1 min.

7. Introduce an appropriate dilution of the anti-mouse BSA sample prepared in phosphate buffer (0.1 M, pH 7.4), tape the access holes and incubate for 30 min. Drain the excess analyte solution and wash the channel with phosphate buffer for 1 min.

8. Introduce the goat anti-mouse IgG HRP solution prepared in 0.1 M phosphate buffer (pH 7.4) containing 0.05% (v/v) Tween 20 into the channel and tape the access holes and incubate for 10 min. Remove the excess solution and wash the conduit with phosphate buffer for 1 min.

9. Place the microchip on the epi-fluorescence microscope stage and turn on the heating fan. Using a thermometer, ensure that the air temperature around the microchip reads 37°C.

10. Now introduce the enzyme substrate solution containing 10 μM Amplex Red and 5 μM H_2O_2 in 0.1 M phosphate buffer and seal the access holes with adhesive tapes. Start a timer immediately. Align the relevant microchannel with the 10X microscope objective to make a fluorescence measurement.

11. Open the mechanical shutter to irradiate the channel with the excitation beam from the microscope lamp, take a fluorescence image and then close the shutter immediately. The total beam exposure time should be less than 3 s to prevent photochemical generation of fluorescent species in the enzyme substrate solution. Repeat this process every five minutes to obtain 6 images over a 30 min enzyme reaction period.

12. To quantitate the fluorescence images, open them using Adobe Photoshop software and measure the average intensity drawing a square box with dimensions about a third to half of the channel width located around the center of the microchannel region.

13. To quantitate the assay, plot the recorded fluorescence intensity in the channel region against the enzyme reaction time and fit the data points to a straight line based on a linear regression analysis using the Excel software. Record the slope of this line and the standard deviation in this slope as calculated by Excel. Note that this standard deviation in the slope is based on the disparity between the actual fluorescence readings from the experiment and the best fit line to

them. Interestingly, this quantity has been found to be comparable to the standard deviation estimated based on measuring the rate of signal generation in multiple assay channels. However, because the former approach requires fewer experiments to be performed, we have adopted it for this experimental module. More importantly, the limit of detection (LOD) estimated based on this standard deviation matches very well with the smallest analyte concentration that can be reliably determined using a microchip platform as established in the literature (see references 4 and 5 from the student handout).

14. Now graph the rate of fluorescence generation (slope of the line in step 13) for a sample minus the same quantity for the blank assay against the concentration of the analyte in the sample, i.e., reciprocal of the sample dilution factor. This is the response curve for the microfluidic ELISA.

15. To determine the limit of detection for the assay, evaluate the ratio of the rate of signal generation (slope of the line in step 13) over three times the standard deviation in this quantity (as measured in step 13). Now plot this ratio, referred to as the signal-to-noise ratio, against the analyte concentration in the sample. Fit at least 4 measurements made at the smallest analyte concentrations to a straight line based on a linear regression analysis using the Excel software. Estimate the limit of detection (LOD) for the assay using the expression LOD = $(1 - b) / m$, where m and b refer to the slope and y-intercept of the best fit line to the signal-to-noise ratio measurements.

Liquid Handling in Microchannels

Chemical reagents may be introduced into the microchannel by dispensing them into an appropriate access hole using a high precision pipettor as shown in Figure 3(a) below and

allowing capillary forces to drive the liquid into the conduit. Care must be taken to ensure that there are no air bubbles trapped in the access hole which otherwise may flow into the channel during the filling process. Reagents may be purged from the microchannels through the use of an in-house vacuum supply system as shown in Figure 3(b). Such a system can comprise a portable

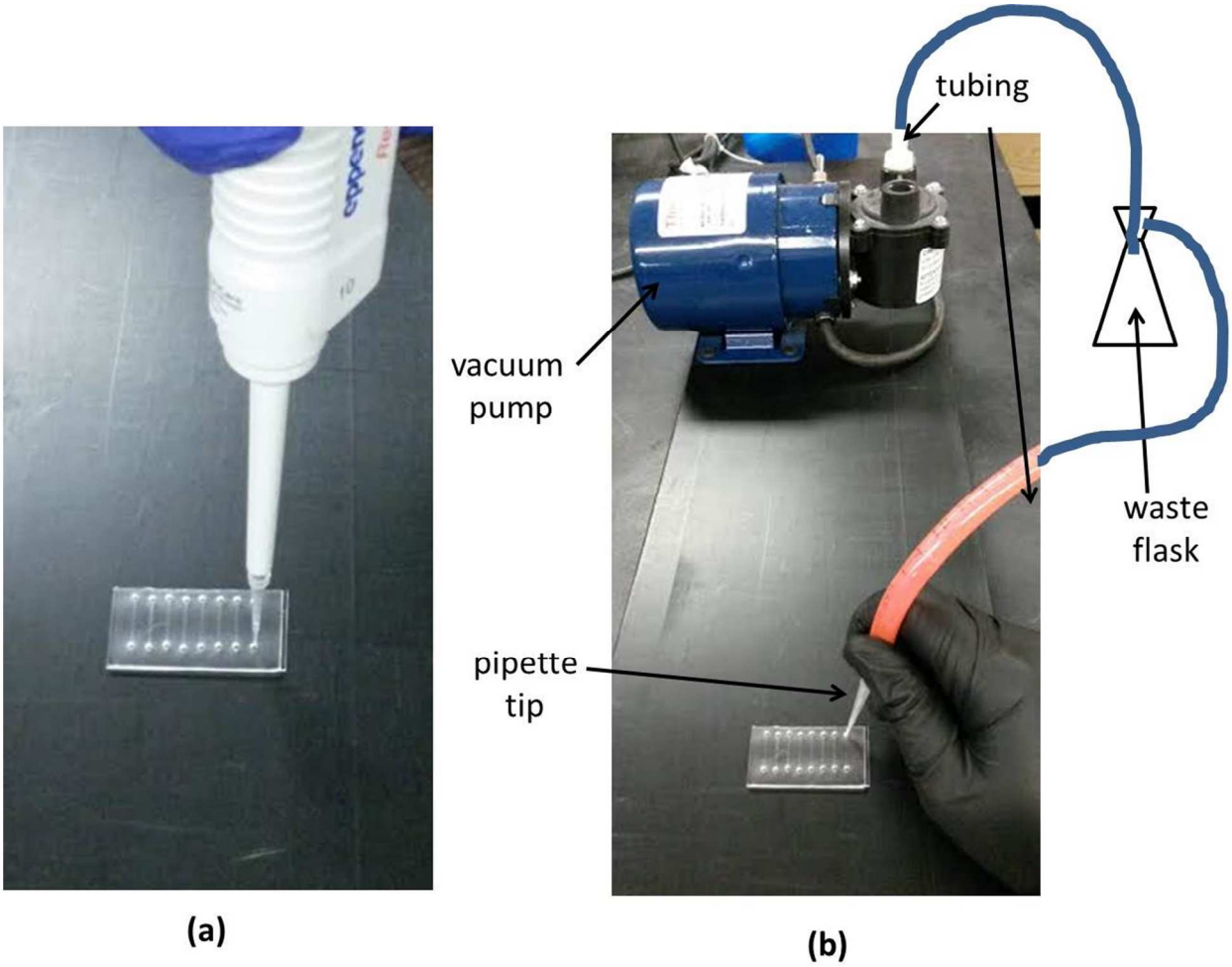

Figure 3: (a) Introduction of chemical reagents into an ELISA microchannel using a high precision pipettor. Notice that the reagent spontaneously flows into the microchannel during this process due to the large capillary force within the conduit. **(b)** Removal of chemical reagents from the ELISA microchannel using a vacuum-pump set-up. The waste flask included in the loop between the pump and the pipette tip prevents any liquid from entering the vacuum pump.

vacuum pump with its vacuum port connected to a pipette tip though a plastic tubing. It is recommended to include a waste flask between the pipette tip and the vacuum port as shown in the figure to prevent any liquid from entering the vacuum pump.

Hazards

Sodium hydroxide is very hazardous in case of skin contact (corrosive, irritant, permeator), eye contact (irritant, corrosive) or ingestion. Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract. Skin contact may produce burns. Inhalation of the spray mist may produce severe irritation of respiratory tract, characterized by coughing, choking, or shortness of breath. Severe over-exposure can result in death. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or occasionally, blistering. (3-aminopropyl)triethoxysilane is corrosive and causes eye and skin burns. This chemical may also lead to severe respiratory and digestive tract irritation with possible burns if inhaled or swallowed. Glutaraldehyde is hazardous in case of skin contact (sensitizer, permeator). Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract. Inhalation of the spray mist may produce severe irritation of respiratory tract, characterized by coughing, choking, or shortness of breath. Severe over-exposure can result in death. Phosphate and carbonate buffers may cause irritation in eyes, skin or the respiratory system. Amplex Red is known to cause only minor discomfort upon contact with skin or eyes but can be harmful if swallowed. Hydrogen peroxide is highly corrosive upon ingestion or contact with skin and eyes. Inhalation of the spray mist may produce severe irritation of respiratory tract, characterized by coughing, choking, or shortness of breath. Prolonged exposure may result in skin burns and ulcerations. Eye protection, hand gloves, and laboratory coats are recommended

while performing this experiment. Contaminated materials should be disposed appropriately as hazardous chemicals. The edges of the glass microfluidic devices pose a small cutting hazard.

Instructor's Notes

Device fabrication

The surface chemistry described in this experimental module is suitable for glass microfluidic devices. Such devices can be made from commercially purchased glass plates that come with a thin layer of chromium and photoresist laid down on one of their surfaces. The fabrication process for the microchips starts with the photolithographic patterning of the desired channel layout on the glass plate using a custom designed photomask. These photomasks can be made by drawing the channel layout using computer-aided design software and printing the file onto a polymer film. Because the desired resolution for the photomask is usually about a few micrometers, it is advisable to print them at a specialized facility. The photolithographic patterning process can be completed using a mask aligner system following procedures provided by the vendor of this equipment. After completion of the photopatterning process, the photoresist layer should be cured in a microposit developer (e.g., MF-319, Rohm and Haas) and the chromium layer removed along the channel network with a chromium etchant. The channels may then be etched to a chosen depth using a solution of buffered oxide etchant purchased from a commercial source. Access holes may be punched into the glass plate at the channel terminals using a micro-abrasive particle blasting system or a drill bit for introducing/purging the ELISA reagents into/out of the microchip. Finally, the microchannels may be sealed off by bringing a glass cover plate in contact with the microchannel plate in de-ionized water and then allowing the two to bond under ambient conditions overnight.[1] It is not advisable to attach external fluidic ports to the access holes in order to minimize the volume of ELISA reagents needed for the

different incubation steps. Solvent evaporation during the incubation periods may be prevented by sealing the access holes with adhesive tapes. While the microfluidic devices used in the current experiments were constructed by the instructor's research laboratory members who have technical expertise in microfabrication methods, these units may also be directly purchased from a commercial vendor[2,3] for a nominal cost in the absence of an in-house microfabrication facility. Moreover, such glass microchips may be produced using alternative approaches[4] requiring minimal fabrication expertise and/or specialized equipment if one is willing to somewhat compromise on the quality of the devices. Finally, it should be possible to adapt the reported ELISA module to polymer microchips that are relatively simple to fabricate.[5,6]

The following ordering information may be used if the microchips are purchased from a commercial vendor.

Microchip dimensions: 2" long and 1" wide

Microchip material: Borosilicate glass

Channel layout: 8 channels laid parallel to each other separated by a center-to-center distance of 5 mm. The array of microchannels should center justified with respect to the edges of the microchip and their axis aligned along the width of the device.

Channel dimensions: 30 μm deep, 500 μm wide and 1.5 cm long

Anticipated cost: 40 USD per microchip device

References

(1) Iles, A.; Oki, A.; Pamme, N. Bonding of soda-lime glass microchips at low temperature *Microfluid. Nanofluid.* **2007**, *3*, 119-122.

(2) Micronit Microfluidics, Company website: http://www.micronit.com/

(3) uFluidix, Company website: http://ufluidix.com/

(4) Yuen, P. K.; Goral, V. N. Low-cost rapid prototyping of whole-glass microfluidic devices *J. Chem. Educ.* **2012**, *89*, 1288-1292.

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(6) Eteshola, E.; Leckband, D. Development and characterization of an ELISA assay in PDMS microfluidic channels. *Sens. Actuators B Chem.* **2001**, *72*, 129-133.

Liquid Handling in Microchannels

Chemical reagents may be introduced into the microchannel by dispensing them into an appropriate access hole using a high precision pipettor as shown in Figure 1(a) below and allowing capillary forces to drive the liquid into the conduit. Care must be taken to ensure that there are no air bubbles trapped in the access hole which otherwise may flow into the channel during the filling process. Reagents may be purged from the microchannels through the use of an in-house vacuum supply system as shown in Figure 1(b). Such a system can comprise a portable vacuum pump with its vacuum port connected to a pipette tip though a plastic tubing. It is recommended to include a waste flask between the pipette tip and the vacuum port as shown in the figure to prevent any liquid from entering the vacuum pump.

Authors' Experimental Data

For reference purposes, experimental data relevant to the quantitation of the reported ELISA module was generated by the authors. In Figure 2, this information has been included to help assess the students' performance in the laboratory. Analysis of this data yielded a limit of

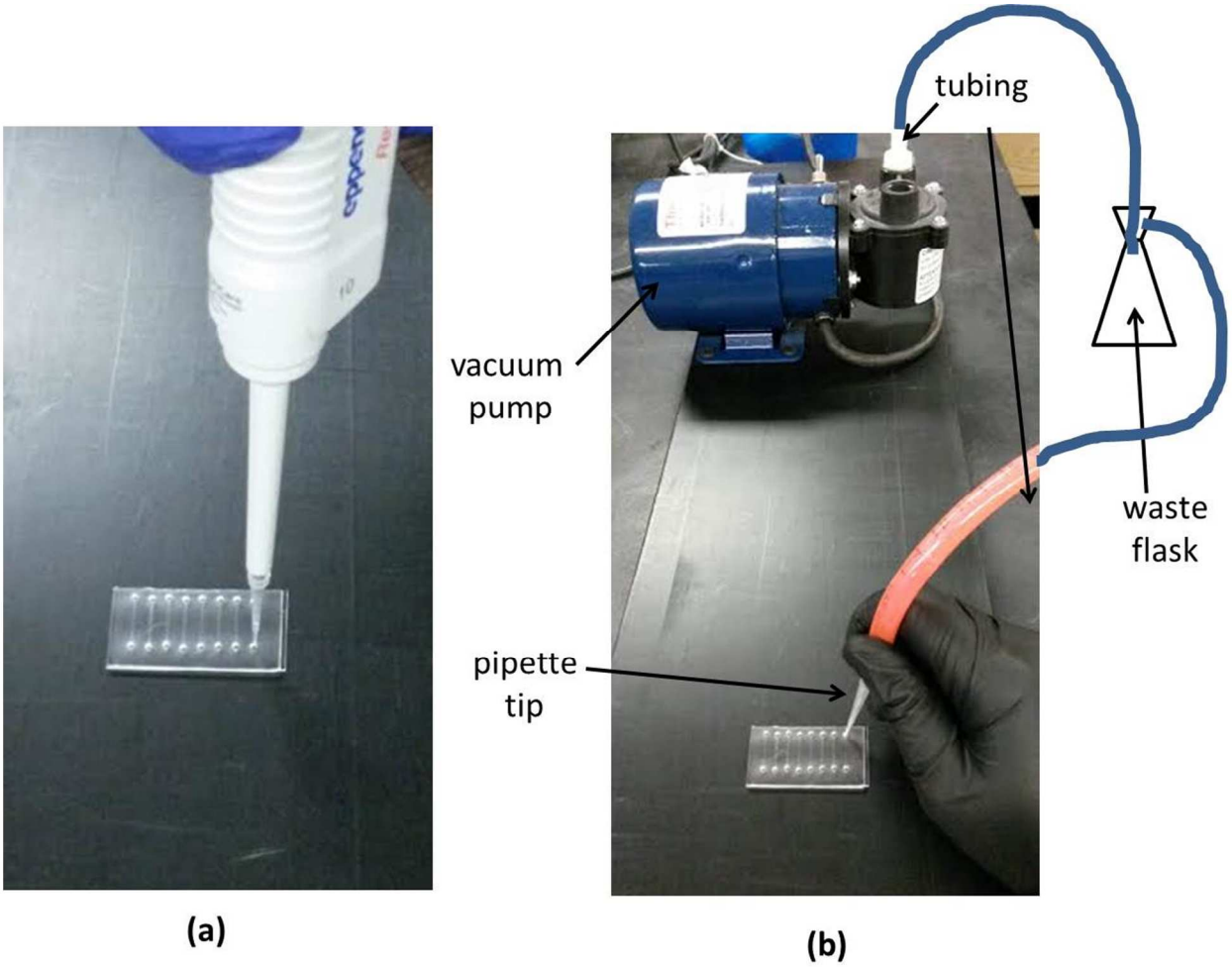

Figure 1: (a) Introduction of chemical reagents into an ELISA microchannel using a high precision pipettor. Notice that the reagent spontaneously flows into the microchannel during this process due to the large capillary force within the conduit. **(b)** Removal of chemical reagents from the ELISA microchannel using a vacuum-pump set-up. The waste flask included in the loop between the pump and the pipette tip prevents any liquid from entering the vacuum pump.

detection (LOD) for the anti-mouse BSA sample of 3.53×10^4 (sample dilution factor) which was within the upper and lower bounds of the reported student data set. Notice that the sample dilution factor was used as a measure for the analyte concentration in our entire analysis as the

supplier for the anti-mouse BSA solution (Sigma-Aldrich) for our experiments was unable to provide any information on the absolute concentration of this protein in the stock sample.

To assess the performance of our microfluidic ELISAs, anti-mouse BSA assays were also carried out on commercial microtiter plates (data not included) for which the LOD value was estimated to be 1.10×10^4 (sample dilution factor). These experiments were carried out on microwell plates purchased from Dynatech Laboratories (Immulon II). To ensure a fair comparison between the microtiter plate and microfluidic ELISAs, the same epi-fluorescence microscope system and data analysis approach were used. For the former case however, BSA was directly laid down onto the surface of the assay compartment by incubating a solution containing 1% (by weight) of this reagent prepared in a 0.1 M carbonate-bicarbonate buffer, pH 9.4, overnight. These assays also required an incubation period of 12 h for the anti-mouse BSA sample, and 1 h each for the detection antibody (goat anti-mouse IgG HRP conjugate). Overall, this corresponded to nearly 28 h of analysis time (including all incubation periods) for the microtiter plate-based experiments as compared to 6 h for the microfluidic assays. The microtiter plate-based ELISAs also consumed 100 μL of sample/reagents per assay which was two orders of magnitude larger than that required in the microfluidic version.

Supplies/Reagents Needed

Reagents/Supplies	**Supplier**	**CAS or Catalog #**
sodium hydroxide	Sigma-Aldrich	1310-73-2
deionized water	Sigma-Aldrich	7732-18-5
methanol	Sigma-Aldrich	67-56-1
(3-aminopropyl)triethoxysilane	Sigma-Aldrich	919-30-2
glutaraldehyde	Sigma-Aldrich	111-30-8

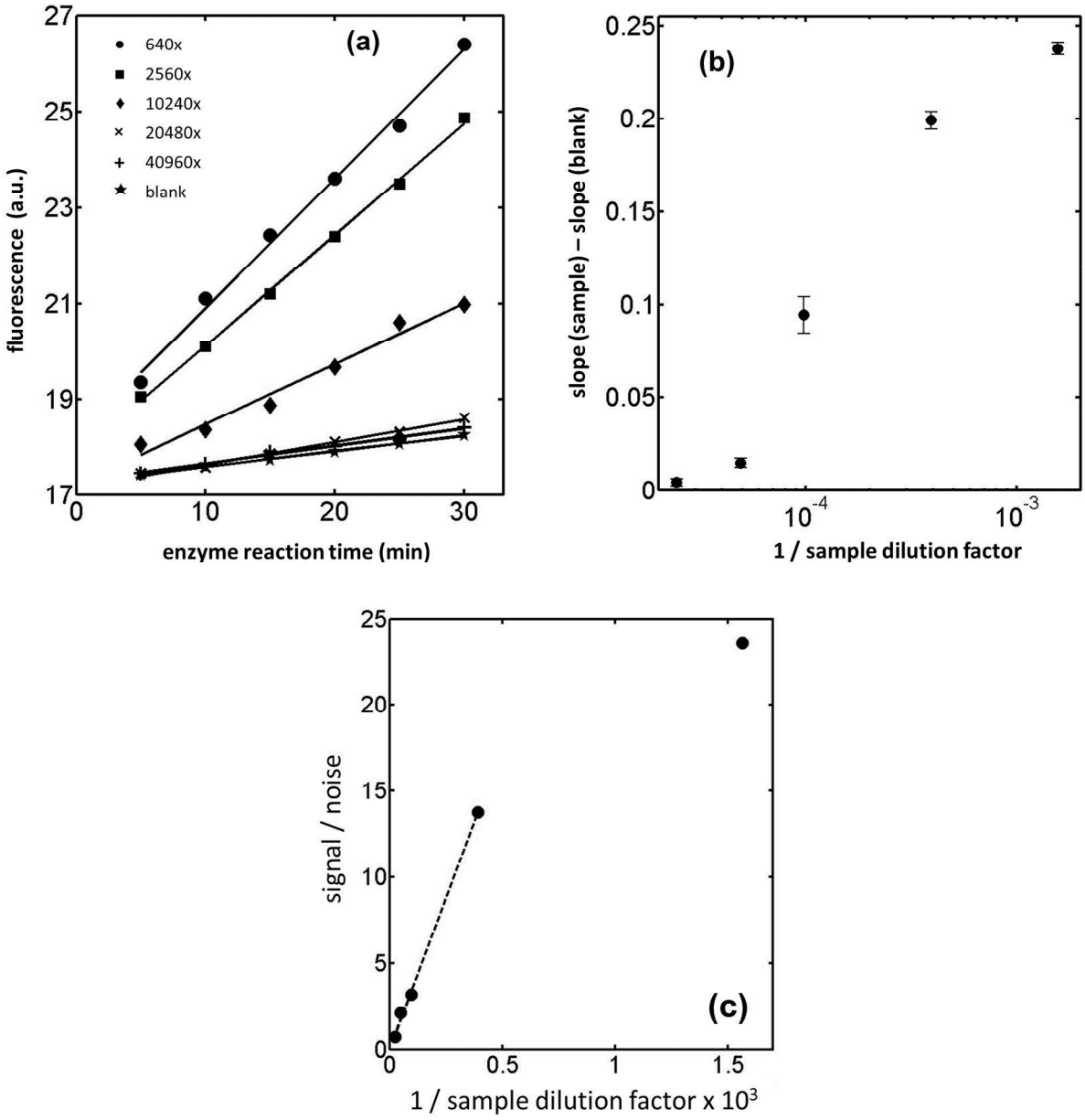

Figure 2: Authors' data on the **(a)** temporal variation in the observed microchannel fluorescence over the enzyme reaction period for different dilutions of the anti-mouse BSA sample **(b)** response curve for the microfluidic ELISA **(c)** estimation of LOD.

bovine serum albumin	Sigma-Aldrich	9048-46-8
sodium carbonate	Sigma-Aldrich	497-19-8

sodium bicarbonate	Sigma-Aldrich	144-55-8
sodium phosphate monobasic	Sigma-Aldrich	10049-21-5
sodium phosphate dibasic	Sigma-Aldrich	7782-85-6
anti-mouse BSA	Sigma-Aldrich	B2901
goat anti-mouse IgG HRP	Sigma-Aldrich	A4416
Tween 20	Sigma-Aldrich	9005-64-5
Amplex red	Sigma-Aldrich	119171-73-2
Hydrogen peroxide	Sigma-Aldrich	7722-84-1
vacuum pump	Thermo Scientific	420-1901
photoresist coated glass plate	Telic Company	4×4×1.65 B270 w/ Cr/ & resist
cover plate	Telic Company	4×4×0.9 B270 clear
resist developer MF-319	Rohm & Haas	10018042
chromium etchant	Transene Company	060-0028000
buffered oxide etchant	Transene Company	060-000BOE
micro-abrasive powder blaster	Vaniman Company	80150
adhesive tape	various	

Sample Assessment Questions and Answers

The following question was used in the course final for fall 2012.

Question: If the recorded fluorescence in an ELISA is 0.76 after 20 min of enzyme reaction and the corresponding value for the background is 0.12, what is the rate of generation of the enzyme reaction product in the assay? Assume that a 1 μM solution of this product species in the assay chamber yields a fluorescence signal of 0.43 and the enzyme reaction follows a zeroth order kinetics.

Answer: Assuming that the measured fluorescence is proportional to the enzyme reaction product concentration, i.e., $I = k[P]$, $k = 0.43/1\ \mu M = 0.43\ \mu M^{-1}$.

Change in fluorescence over a 20 min. period due to generation of the enzyme reaction product = recorded fluorescence – background value = 0.76 – 0.12 = 0.64.

Therefore, the rate of generation of enzyme reaction product = 0.64/20/k = 0.0744 μM/min

The following question was used in the course final for fall 2013.

Question: If an enzyme substrate is consumed at a rate of 2.3 μM/min at saturation kinetics and this rate of consumption drops by a factor 5 when the substrate concentration is reduced to 3.1 μM, determine the Michaelis-Menten rate equation for the enzyme.

Answer: The Michaelis-Menten rate equation is given by

$$-\frac{d[S]}{dt} = \frac{\vartheta_{max}[S]}{K_m + [S]}$$

where $\vartheta_{max} = 2.3\ \mu M/min$ in the current problem. Now from the condition when $[S] = 3.1\ \mu M$, $-d[S]/dt = 0.2 \times 2.3\ \mu M/min$ = 0.46 μM/min, one can determine $K_m = 12.4\ \mu M$. The Michaelis-Menten rate equation for the present enzyme therefore is

$$-\frac{d[S]}{dt} = \frac{2.3[S]}{12.4 + [S]}\ \mu M/min$$

where $[S]$ is the enzyme substrate concentration expressed in μM.